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14. ABSTRACT

These studies were conducted to determine the function of DACH1 in regulating prostate cancer growth in vitro and in vivo, and to determine the normal function of DACH1 in prostate development. We demonstrated that DACH1 suppresses prostate cancer cellular growth induced by ErbB2. We demonstrated similar mechanisms govern DACH1 restrain of prostate tumorigenesis promoted by Ras, c-Myc and c-Src. We identified the key molecular targets regulated by DACH1 in vitro and in vivo and showed using ChIP analysis the binding of DACH1 to key target genes. We used genetic deletion studies to identify the key function for DACH1 in restraining cytokine secretion. IL-8 and IL-6 are the key cytokines demonstrated to promote prostate cancer growth and we showed that DACH1 is the key endogenous mechanism governing restraint of prostate epithelial cell cytokine secretion (IL-8 and IL-6).

15. SUBJECT TERMS

Retinal determination gene network (RDGN) pathway, *dachshund*, *prostate cancer*, *androgen receptor (AR)*.

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Table of Contents

INTRODUCTION

Prostate cancer is the most frequent malignancy and the second leading cause of cancer-related death among men in the United States (1, 2). The retinal determination gene network (RDGN) pathway, consisting of the *dachshund* (*dac*), *eyes absent* (*eya*), *eyeless*, and *sine oculis* (*so*) (*Six*) genes, regulates cell fate determination in metazoans and is essential for retinal, kidney, and limb development on mouse (5, 7, 8). Recently, we reported that expression of DACH1 is lost in human prostate cancer tissues and restoration of DACH1 inhibited ligand induced AR activity (18, 19). Although the abnormal expressions of RDGN genes have been reported in prostate cancer, the precise role of RDGN in prostate cancer is not clear. We aimed to determine the role of DACH1 in prostate cancer cellular growth and the role of Dach1 in prostate gland development and tumor progression.

KEYWORDS

Retinal determination gene network (RDGN) pathway, *dachshund, prostate cancer, androgen receptor (AR).*

OVERALL PROJECT SUMMARY

These studies were conducted to determine the function of DACH1 in regulating prostate cancer growth in vitro and in vivo, and to determine the normal function of DACH1 in prostate development. We demonstrated that DACH1 suppresses prostate cancer cellular growth induced by ErbB2. We demonstrated similar mechanisms govern DACH1 restrain of prostate tumorigenesis promoted by Ras, c-Myc and c-Src. We identified the key molecular targets regulated by DACH1 in vitro and in vivo and showed using ChIP analysis the binding of DACH1 to key target genes. We used genetic deletion studies to identify the key function for DACH1 in restraining cytokine secretion. IL-8 and IL-6 are the key cytokines demonstrated to promote prostate cancer growth and we showed that DACH1 is the key endogenous mechanism governing restraint of prostate epithelial cell cytokine secretion (IL-8 and IL-6).

1. To evaluate the physiological role of endogenous DACH1 in an ErbB2 induced prostate tumor model;
2. To examine the role of DACH1/Eya1/Six1 in prostate cancer cell AR signaling transduction, proliferation, migration and invasion *in vitro*;
3. To investigate the role of DACH1/eya/Six1 in tumor growth *in vivo* using xenograft models
4. To analyze the expression of DACH1, Eya1 and Six1 during the process of human prostate tumor development.

BODY

Aim 1. Evaluate the physiological role of DACH1 in prostate gland development and ErbB2-induced prostate tumor. Investigating the role of DACH1 as a physiological co-repressor of AR will be conducted on transgenic mice in which the *Dach1* gene is flanked by loxP sites (*Dach1*$^{fl/fl}$) and crossed with Probasin-Cre (Pb- Cre) to generate double transgenic mice, those mice will be crossed with Probasin-erbB2Δ (Pb-erbB2) transgenic mice to create triple transgenic mice, *Dach1*$^{fl/fl}$/Pb-Cre/ Pb-erbB2.

Conditional *Dach1* gene knockout in the prostate demonstrates a role for endogenous Dach1 as an inhibitor of proliferation via cyclin D1. In our previous report we showed a reduction in Ki67 staining and reduction in apoptosis using TUNEL staining in the *Dach1*$^{-/-}$ epithelial cells. In order to examine the mechanisms by which Dach1 regulated apoptosis and cellular proliferation in the prostate we conducted immunohistochemical staining for the cell cycle proteins cyclin D1 and cyclin A1. The abundance of these two cyclins was substantially increased in the Dach1$^{-/-}$ prostate epithelial cells in both the ventral and anterior prostate (Fig. 1A-H).

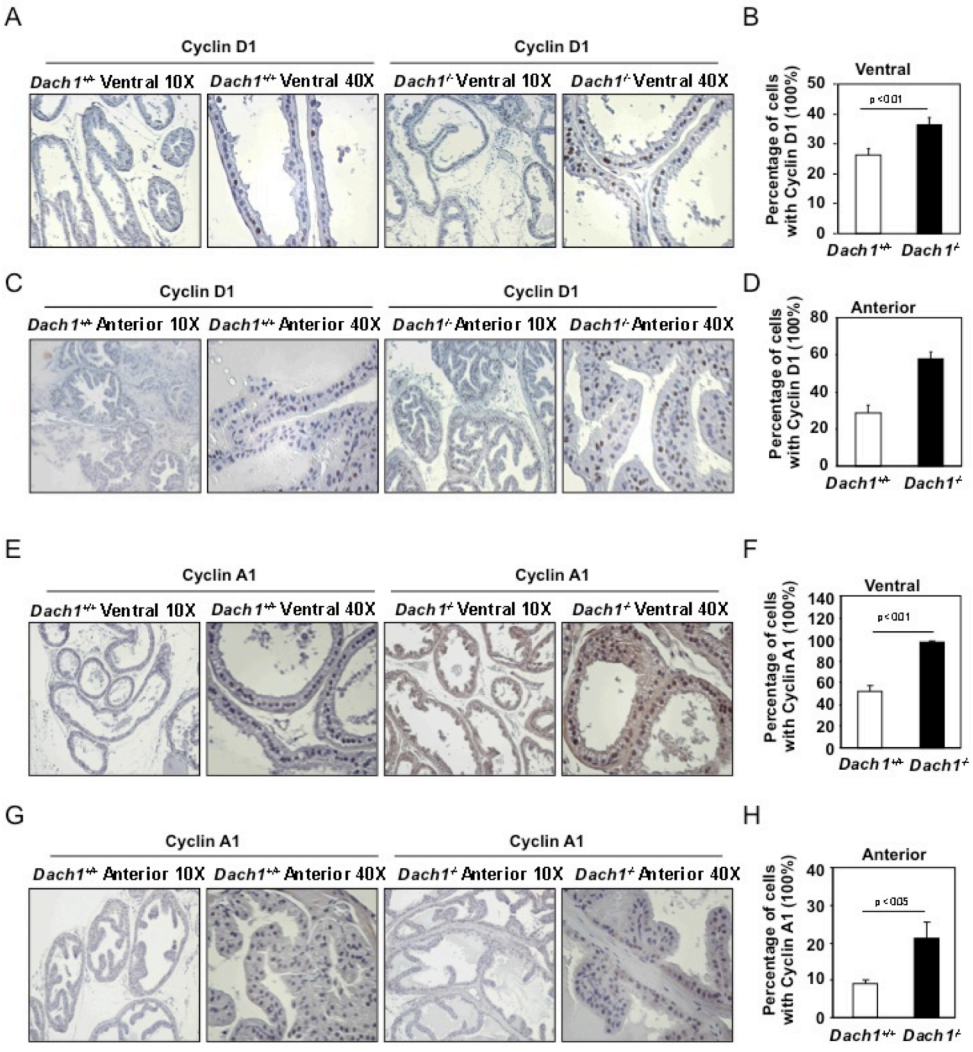

Figure 1. Prostate specific expression of *Dach1* enhances cyclin D1 and cyclin A1. A-D) Immunohistochemical staining for cyclin D1 in ventral and anterior prostate of bi-transgenic mice encoding *Dach1*$^{fl/fl}$/Probasin-Cre mice. E-H) Immunohistochemical staining for cyclin A1 in ventral and anterior prostate of bi-transgenic mice encoding *Dach1*$^{fl/fl}$/Probasin-Cre mice.

Aim 2. Examine the role of DACH1 in prostate cancer cell AR signaling transduction, proliferation, migration and invasion *in vitro:*

We next examined the role of DACH1 to restrain AR negative and AR positive prostate cancer cell contact independent growth. In PC3 cells DACH1 inhibited colony formation (Fig.2a) requiring the DS domain of DACH1. The predominant effect of DACH1 was to reduce the number of new colonies, consistent with a role for DACH1 to restrain prostate cancer stem cells that give rise to new colonies. We next examined the effect of DACH1 on the proliferation of the AR+ ve C4-2 cells. The C4-2 prostate cancer cells are a well characterized model of androgen-independent prostate cancer. The PI3K pathway is constitutively active in C4-2 due to the loss of the tumor suppressor PTEN, which is also deleted or inactivated in up to 70% of advanced androgen-independent prostate cancers. DACH1 or the DACH1 ΔDS domain mutant was expressed in the C4-2 cells (Fig. 2D), as evidenced by IHC to the DACH1 protein. Western blot analysis to the N-terminal tag of DACH1 demonstrated similar levels of the exogenous DACH1 or DACH1 ΔDS proteins (Fig. 2D). Expression of DACH1 reduced C4-2 cellular proliferation as assessed by the MTT assay and cell counting (Fig. 2E and F). DACH1 reduced proliferation approximately 50% at 6 days.

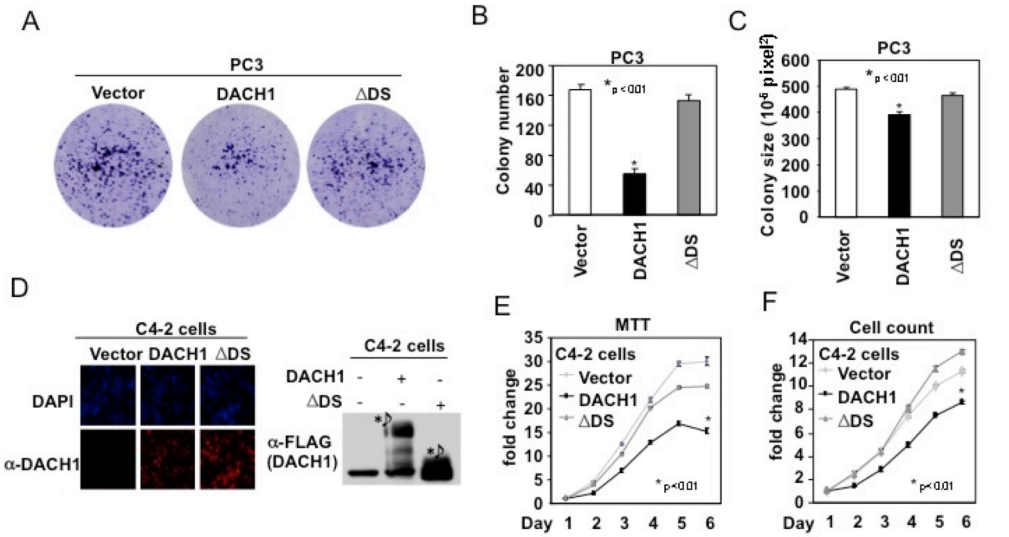

Figure 2. DACH1 inhibits AR-negative prostate cancer cell proliferation and contact independent growth by the DS domain. A) Colony forming assays were conducted with PC3 stable cell lines expressing control vector, DACH1 or ΔDS with colonies stained using crystal violet and B) colony number or C) colony size determined using N>5 separate experiments. D) C4-2 cells expressing either control vector DACH1 or the DACH1 ΔDS mutant were assessed for DACH1 abundance by immuno-histochemistry. DAPI and immunofluorescence for DACH1 is shown. Western blot is shown of the cells with an antibody directed to the N-terminal FLAG tag. E) The cellular proliferation rate of C4-2 cells expressing DACH1 or mutant DS was determined by either MTT assay or F) cell counting. Data are mean ±SEM for N>5 throughout.

Aim 3: Investigate the role of DACH1 in tumor growth *in vivo*.

In our previous studies we showed that DACH1 inhibition of prostate cancer cellular invasion and migration required CXCL gene expression. DACH1 expression was reduced in metastatic human prostate cancer. To determine whether endogenous DACH1 regulated the secre-tion of the cytokine signaling mRNA module identified in human prostate cancer cells in tissue culture, the prostatic epithelium of the bitransgenic mice (Dach1$^{fl/fl}$ probasin Cre) was analyzed. A cytokine array analysis demonstrated increased secretion of CXCL signaling in the *Dach1*$^{fl/fl}$Probasin-Cre bitransgenic mice PECs in culture. The increased abundance of IL6 and KC (homolog of human IL8) in *Dach1*$^{-/-}$ PEC was confirmed by quantitative ELISA (Fig. 3A and B). The abundance of IL6 was increased approximately 1,000 -fold (Fig. 3A) and the abundance of KC was also increased by approximately 1,000-fold (Fig. 3B) when assayed by ELISA. The *Dach1*$^{-/-}$ PEC derived from the anterior prostate showed a similar increase in cellular migratory distance and velocity. The mRNA levels for IL6 and IL8 determined by quantitative PCR were increased by approximately 6- fold in *Dach1*$^{-/-}$ versus Dach1 *Dach1*$^{+/+}$ PEC.

The circulating serum levels for IL6 and IL8 determined by quantitative ELISA in the Dach1 wild-type versus knockout mice show a 3-fold difference in abundance (Fig. 3D and E). The *Dach1*$^{-/-}$ PEC of the ventral prostate showed an approximately 250% increase in both cellular migratory distance (data not shown) and velocity (Fig. 3C). The addition of media from the *Dach1*$^{-/-}$ PEC enhanced migration of *Dach*$^{+/+}$ PEC (Fig. 3C, lanes 1 vs. 2) and media from *Dach1*$^{+/+}$ PEC reduced migration of *Dach1*$^{-/-}$ PEC (Fig. 3C, lanes 9 vs. 10). This finding suggested that endogenous Dach1 inhibits the secretion of factors that promote PEC cellular migration (Fig. 3F). The addition of IL6 or CXCLI (KC, murine homolog of IL8) enhanced migration of *Dach1*$^{-/-}$ PEC. Addition of an immunoneutralizing antibody to IL6 or CXCLI reduced the migration of *Dach*$^{-+/+}$ PEC (Fig. 3C).

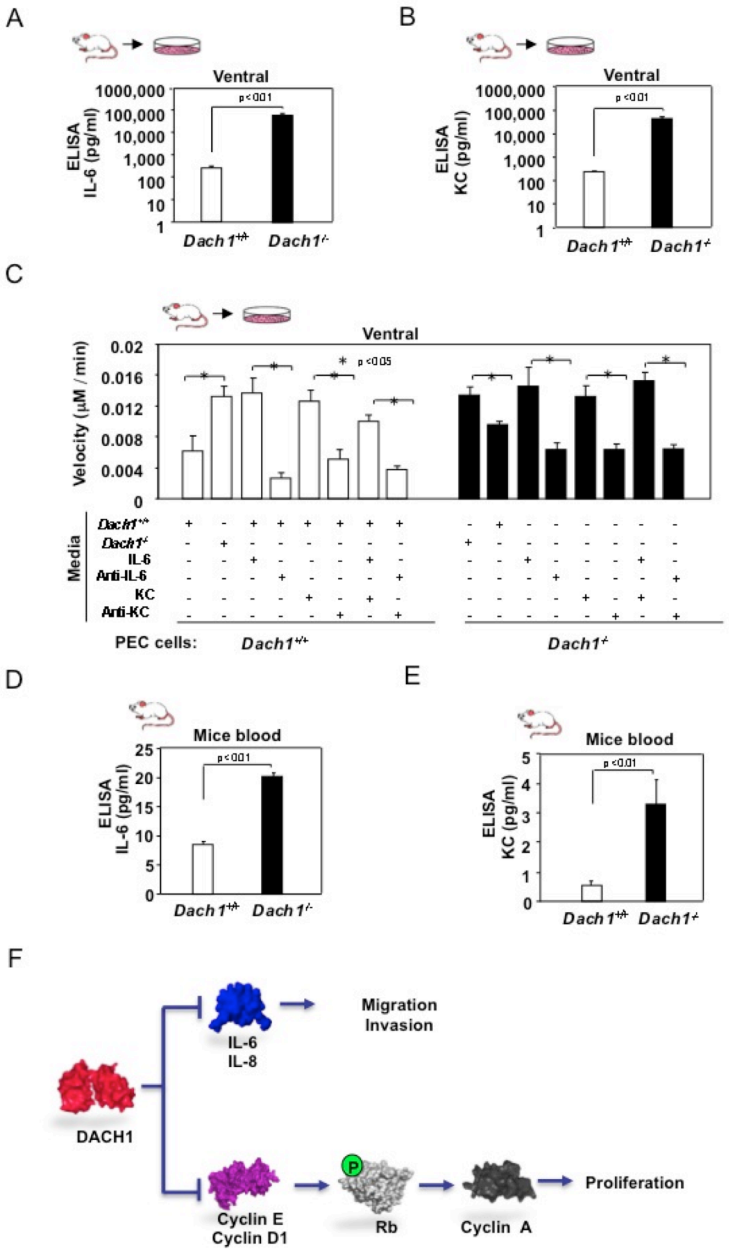

Figure 3. *Dach1* is a dominant endogenous restraint of prostate epithelial cell cytokine production and thereby cellular migration. The *Dach1*$^{fl/fl}$ Probasin-Cre prostate epithelial cells were cultured and comparison was made to *Dach1*$^{+/+}$. A) ELISA was used to determine the relative abundance of cytokines in the supernatant (ventral prostate epithelial cells) in culture for IL-6 and B) KC in pg/mL (Note: Log scale). C) Analysis of migratory cell velocity for prostate epithelial cells derived from *Dach1*$^{+/+}$ or *Dach1*$^{-/-}$ PECs. Cells were co-incubated with media derived from either *Dach1*$^{+/+}$ or *Dach1*$^{-/-}$, incubated with IL-6 or anti-IL-6 antibody. KC or anti-KC antibody is indicated in the Figure. P value is indicated (significance <0 .05). Data is shown as mean ± SEM of three separate experiments. D) ELISA was used to determine the relative abundance for cytokines in the *Dach1* wild-type vs. knockout mice in circulating serum of IL-6 and E) KC in pg/mL (Note: Log scale). F) Schematic representation of *Dach1* as key determinant of prostate cellular cytokine secretion and cellular proliferation.

Aim 4. Analyze the expression of DACH1, Eya1 and Six1 in human prostate tumor samples.

We had previously begun an investigation to determine whether a clinical correlation existed for reduction of DACH1 expression in metastatic prostate cancer. In our previous studies of clinical databases we demonstrated the relative abundance of DACH1 was reduced in prostate cancer compared with benign prostate disease, with significant further reduction in metastatic prostate cancer samples. As we had shown in tissue culture that Dach1 repressed IL-6 and IL-8 we examined the relative abundance of these two parameters in the same prostate cancer sample of individual patients (Fig 4. A,C). We then displayed the data in a 2 dimensional matrix (Fig. 4B,D). Consistent with the model in which DACH1 repressed expression of IL-8 and IL-6 in matched individual samples there was a substantial increase in the quadrant in which low DACH1 expression correlated with high IL-8 or high IL-6 expression (Fig. 4 B,D).

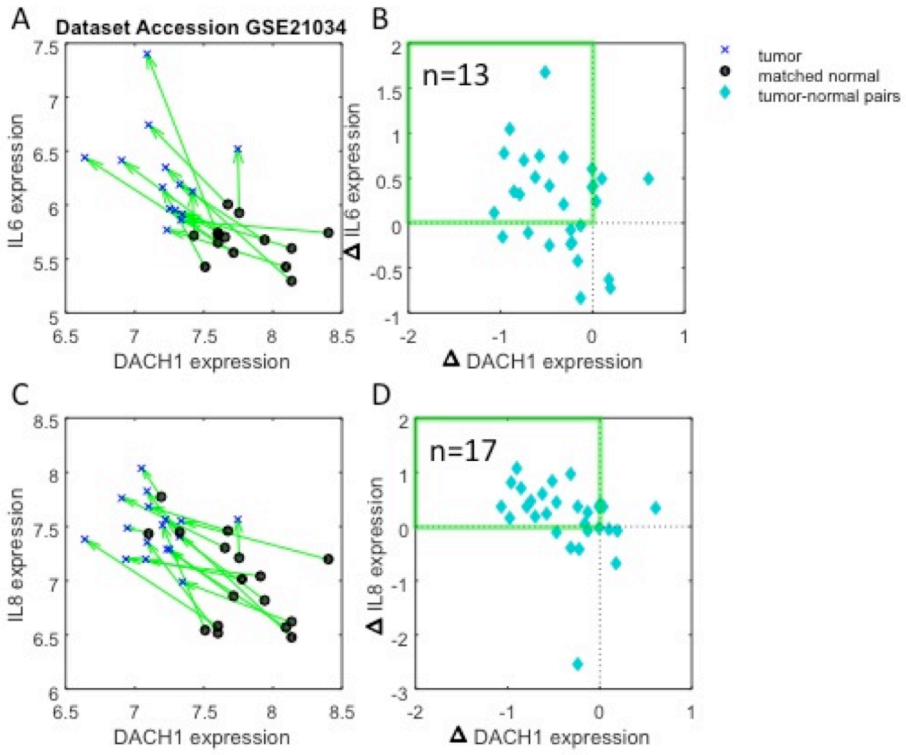

Figure 4. **The relationship between DACH1 and IL-6, IL-8 in metastatic prostate cancer.** Gene expression data for individual patients is linked by green lines shown for (A) IL6 or (C) IL-8 w ith normalized data shown in 2 D grid (B,D)

KEY RESEARCH ACCOMPLISHMENTS

- Endogenous Dach1 was shown to be the key restraint for prostate cytokine secretion in normal prostate using Cre excision of Dach1 in transgenic mouse prostate (genetic deletion of Dach1 in the prostate reduced Il-8 and Il-6 by 10,000 fold) (Chen et al. Cancer Res 2015, May).
- Endogenous Dach1 was shown to convey restrain to prostate epithelial cell proliferation in vivo using Ki67 immunostaining
- Expression of Dach1 was shown to block prostate cancer cell proliferation in tissue culture and tumor growth in vivo in both androgen receptor positive and AR negative prostate cancer cell lines.
- DACH1 restrained cytokines and chemokines in prostate cancer cells
- DACH1 restrained prostate cancer cell migration via IL-6 and IL8
- Mutations of DACH1 were identified in human kidney disease, which altered expression of TGFb (Schild et al 2013 below)

CONCLUSION

Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

The current studies provide fundamental new information about the mechanisms restraining IL-8 and IL-6 secretion in the prostate by identifying DACH1 as the key mechanism. IL-8 and IL-6 have been linked to prostate cancer risk and prostate cancer progression however the mechanisms that lead to increased secretion of IL-8 and IL-6 were previously unknown. As we showed DACH1 expression is lost in prostate cancer progression, DACH1 may function in part as a s tumor suppressor through restraining cytokine secretion.

Future studies will examine the role of Eya and Six in this regulatory function of DACH1 and to continue the studies with the transgenic mice as originally proposed.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Peer-Reviewed Scientific Journals

- Schild R, Knuppel T, Konrad M, Bergmann C, Trautmann A, Kemper M, Wu K, Yaklichkin S, Wang J, Pestell RG, Muller-Wiefel DE, Schaefer F, Weber S. Double homozygous missense mutations in DACH1 and BMP4 in a patient with bilateral cystic renal dysplasia. Nephrol. Dial. Transplant. 2013 Jan;28(1):227-32. Epub 2012 Dec 21.

- Chen K, Wu K, Wang L, Jiao X, Ju X, Li Z, Ertel A, Addya S, McCue P, Lisanti MP, Wang C, Davis RJ, Mardon G, Pestell RG. The Endogenous Cell-Fate Factor Dachshund Restrains Prostate Epithelial Cell Migration via Repression of Cytokine Secretion via a CXCL Signaling Module.. Cancer Res. 2015 May 15;75(10):1992-2004

Invited Articles

- Velasco-Velázquez MA, Wu K, Loro E, Pestell RG. “Inhibition of Breast Tumor Stem Cells Expansion by the Endogenous Cell Fate Determination Factor Dachshund.” Chapter in Volume 6 of: Stem Cells and Cancer Stem Cells: Therapeutic Applications in Disease and Injury. (In Press)

Abstracts
List presentations made during the last year

- Chen K, Wu K, Zhang W, Zhou J, Stanek TS, Li Z, Wang C, Shirley LA, Rui H, McMahon S, Pestell RG. A p53-dependent G2/M checkpoint governed by the cell-fate factor Dachshund in non-small cell lung cancer. AACR 103rd Annual Meeting, March 31 – April 4, 2012, Chicago, IL.
- Li Z, Hu J, Chen K, Wu J, Pestell RG. DACH1 inhibited prostate cancer cellular proliferation and Interleukon-6 signaling. AACR 103rd Annual Meeting, March 31 – April 4, 2012, Chicago, IL.
- Wang J, Cai S, Chen K, Sun Y, Li S, Pestell RG, Wu K. Regulation of AR

transcriptional activity and prostate cancer cellular proliferation by DACH1/Eya1/Six1 pathway. AACR Annual Meeting, April 6-10, 2013, Washington, DC.

- Pestell RG, Wu K, Chen K, Wang C, Jiao X, Wang J, Cai S, Addya S, Sorensen P, Lisanti M, Quong A, Ertel A. The Cell Fate Factor DACH1 Represses YB-1- mediated Oncogenic Transcription and Translation. San Antonio Breast Cancer Symposium, December 10-14, 2013. San Antonio, Texas.
- Pestell RG, Chen K, Wu K, Gormley M, Ertel A, Zhang W, Zhou J, DiSante G, Li Z, Rui H, Quong A, McMahon S, Deng H, Lisanti M, Wang C, Post-translational Modification of the Cell-Fate Factor Dachshund Determines p53 Binding and Signaling Modules in Breast Cancer, San Antonio Breast Cancer Symposium, December 10-14, 2013, San Antonio, Texas.

INVENTIONS, PATENTS AND LICENSES

- Invention disclosure to Thomas Jefferson DACH1 Regulates Stem Cells (TJU Ref: PES_RIC.004)

REPORTABLE OUTCOMES

1. Research product: Bi transgenic mice which delete DACH1 in prostate epithelial cells (Probasin-Cre/Dach1 $^{fl/fl}$
2. Prostate cancer cell lines were generated (PC3-DACH1, PC3-DACH1 ΔDS)
3. Scientific discovery that: DACH1 suppresses prostate cancer cellular growth induced by ErbB2.
4. Scientific discovery that: Dach1 restrains prostate tumorigenesis promoted by Ras, c-Myc and c-Src.
5. Scientific discovery that: We identified the key molecular targets regulated by DACH1 in vitro and in vivo and showed using ChIP analysis the binding of DACH1 to key target genes.
6. Scientific discovery that: We used genetic deletion studies to identify the key function for DACH1 in restraining cytokine secretion. IL-8 and IL-6 are the key cytokines demonstrated to promote prostate cancer growth and we showed that DACH1 is the key endogenous mechanism governing restraint of prostate epithelial cell cytokine secretion (IL-8 and IL-6).

OTHER ACHIEVEMENTS

1. Bi transgenic mice were generated Probasin-Cre/Dach1$^{fl/fl}$
2. Prostate cancer cell lines were generated (PC3-DACH1, PC3- DACH1 ΔDS)
3. Submitted RO1 grant application

NIH R01 CA 086072-12 (Pestell)
03/01/00 - 08/28/14
$386,250/yr (Total $1,931,250)
Androgen Receptor Function in Prostate Cancer

Specific Aim: To determine the role of cyclin D1 and androgen receptor mutations in prostate cancer cellular growth.

REFERENCES

- Schild R, Knuppel T, Konrad M, Bergmann C, Trautmann A, Kemper M, Wu K, Yaklichkin S, Wang J, Pestell RG, Muller-Wiefel DE, Schaefer F, Weber S. Double homozygous missense mutations in DACH1 and BMP4 in a patient with bilateral cystic renal dysplasia. Nephrol. Dial. Transplant. 2013 Jan;28(1):227-32. Epub 2012 Dec 21.
- Chen K, Wu K, Wang L, Jiao X, Ju X, Li Z, Ertel A, Addya S, McCue P, Lisanti MP, Wang C, Davis RJ, Mardon G, Pestell RG. The Endogenous Cell-Fate Factor Dachshund Restrains Prostate Epithelial Cell Migration via Repression of Cytokine Secretion via a CXCL Signaling Module.. Cancer Res. 2015 May 15;75(10):1992-2004